RESEARCH ARTICLE

Diversity and distribution of cytochrome oxidase I (COI) haplotypes of the brown marmorated stink bug, *Halyomorpha halys* Stål (Hemiptera, Pentatomidae), along the eastern front of its invasive range in Eurasia

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Abstract

The arrival, establishment and pest status of *Halyomorpha halys* in Europe and non-native countries in Asia have been well-documented, with thorough characterisation of the genetic diversity and occurrence of cytochrome oxidase I (COI) haplotypes in Switzerland, France, Hungary, Italy and Greece. However, a number of gaps exist in terms of the characterisation of the haplotype diversity and occurrence of *H. halys* along the invasion front that covers eastern Europe, western and central Asia. To contribute towards filling this gap, the COI haplotype diversity and distribution were investigated for *H. halys* collected in Serbia, Ukraine, Russia, Georgia and Kazakhstan. A total of 646 specimens were analysed and five haplotypes were found (H1, H3, H8, H33 and H80). Haplotype H1 was present in all five countries investigated and was the only haplotype detected amongst > 500 specimens collected from Ukraine, Russia and Georgia. H1 (82%) was the dominant haplotype found in Kazakhstan, alongside H3 (18%). In contrast to the low or no diversity observed in these four countries, Serbia had higher haplotype diversity and was repre-

sented by five haplotypes. Although H3 was dominant (47%) in Serbia, H1 was also prevalent (40%); the remaining haplotypes (H8, H33 and H80) were minor contributors (1–11%) to the haplotype composition. The results are discussed in context with other known populations in neighbouring countries and patterns of haplotype diversity indicate the movement of successful invasive populations in Europe to generate secondary invasions along the eastern front of the invasion in Eurasia. Possible scenarios regarding the spread of particular haplotypes in these regions are discussed, along with suggestions for future research to fill existing gaps.

Introduction

Halyomorpha halys Stål (Hemiptera: Pentatomidae) is native to East Asia [China (including Taiwan), Japan, Korea, Myanmar and Vietnam; Wang and Liu 2005], but has become invasive in a number of locations in North America, South America and Eurasia (Hoebeke and Carter 2003; Leskey et al. 2012; Haye et al. 2015; Gapon 2016; Faúndez and Rider 2017; Hamilton et al. 2018). In many of these locations, this highly polyphagous insect has become a serious economic pest of agricultural crops, including field, fruit, vegetable and nut crops (Pansa et al. 2013; Rice et al. 2014; Haye et al. 2015; Maistrello et al. 2017; Bosco et al. 2018; Hamilton et al. 2018; Musolin et al. 2018). The global spread of *H. halys* has largely been facilitated through travel, transportation and commercial trade, as the pest gains entry into new locations as a stowaway inside containers, vehicles, packaging and luggage (Hamilton et al. 2018). This poses a significant risk of spread when invasive species establish in a region with a significant transportation hub (Bacon et al. 2012) and, in fact, Turbelin et al. (2017) state that the unintentional ingress of invasive alien species is largely due to trade and ignorant possessions, including stowaways, as well as assisted transport in motor vehicles, trains, boats and planes.

The arrival and establishment of *H. halys* in Europe and non-native areas of Asia has been well-documented (see timeline in Table 1), with the first invasive populations established in Liechtenstein (2004) and Switzerland (2007). Populations expanded rapidly throughout several Swiss Cantons (Haye et al. 2014) and were found in neighbouring Germany and France within five years (Table 1). Prior to 2011, populations of *H. halys* were only known from western European countries; however, the discovery of the pest in Greece in 2011 (Milonas and Partsinevelos 2014) and Italy in 2012 (Maistrello et al. 2014) signalled the arrival of the pest in southern Europe as well (Table 1). The first establishment in eastern Europe was documented in 2013, when *H. halys* was found in Sochi, Russia (Mityushev 2016), with subsequent spread into western Asia in 2015 as the pest moved within the Caucasus region into Georgia (Gapon 2016; Musolin et al. 2018; Table 1), where populations increased rapidly from 2015 to 2016

Table I. Timeline of the establishment of *Halyomorpha halys* in invaded areas of Eurasia, including western Europe, southern Europe, eastern Europe, western Asia, and central Asia.

Year	Country	Geographical region*	Reference
2004	Liechtenstein	western Europe	Arnold 2009
2007	Switzerland	western Europe	Wermelinger et al. 2008
2011	Belgium	western Europe	Claerebout et al. 2018
	Germany	western Europe	Heckmann 2012
	Greece	southern Europe	Milonas and Partsinevelos 2014
2012	France	western Europe	Callot and Brua 2013
	Italy	southern Europe	Maistrello et al. 2014
2013	Russia	eastern Europe	Mityushev 2016
2014	Hungary	eastern Europe	Vétek et al. 2014
2015	Austria	western Europe	Rabitsch and Friebe 2015
	Serbia	southern Europe	Šeat 2015
	Romania	eastern Europe	Macavei et al. 2015
	Georgia and Abkhazia	western Asia	Gapon 2016; Musolin et al. 2018
2016	Bulgaria	eastern Europe	Simov 2016
	Kazakhstan	central Asia	Temreshev et al. 2018
	Slovakia	eastern Europe	Hemala and Kment 2017
	Spain	southern Europe	Diolo et al. 2016
	Ukraine	eastern Europe	Uzhevskaya 2017
2017	Azerbaijan	western Asia	Nuriyeva 2018, 2020
	Croatia	southern Europe	Šapina and Jelaska 2018
	Slovenia	southern Europe	Rot et al. 2018
	Turkey	western Asia	Güncan and Gümüş 2019
2018	Albania	southern Europe	Claerebout et al. 2018
	Bosnia and Herzegovina	southern Europe	Zovko et al. 2019
	Czech Republic	eastern Europe	Kment and Březíková 2018
	Malta	southern Europe	Tassini and Mifsud 2019
	Poland	eastern Europe	Claerebout et al. 2018; Bury 2021
	Portugal	southern Europe	Grosso-Silva et al. 2020
2019	Moldova	eastern Europe	Derjanschi and Chimişliu 2019
	North Macedonia	southern Europe	Konjević 2020

* Geographic regions as defined by the United Nations online publication "Standard country codes for statistical use"; https://unstats.un.org/unsd/methodology/m49/

(Bosco et al. 2018). In 2016, *H. halys* was found in Kazakhstan (Esenbekova 2017; Temreshev et al. 2018), thereby indicating the first population in central Asia. The documented establishment in each country within each region in Europe and non-native areas in Asia is shown in Table 1. Southern and eastern Europe have seen a great deal of expansion of *H. halys* populations since they were first documented in these regions. From 2015 to 2019, an additional nine southern European countries (Serbia, Spain, Croatia, Slovenia, Bosnia and Herzegovina, Albania, Malta, Portugal and North Macedonia; Table 1) have documented the establishment of *H. halys*. Similarly, following the initial establishment in Russia, eight eastern European countries have documented *H. halys* populations from 2014 to 2019 (Hungary, Romania, Bulgaria, Slovakia, Ukraine, Czech Republic, Poland and Moldova; Table 1). The explosion of new populations in recent years suggests the continued movement and spread of *H. halys* in non-native areas of Eurasia. Already, observations in the Caucasus region suggest the occurrence of 2 or 3 generations per year, allowing substantial numbers to build during

a single growing season and likely resulting in the severe agricultural damage observed in Russia and Georgia (Musolin et al. 2018, 2019). Similarly, the population density of *H. halys* in agricultural areas in Greece and Turkey is increasingly high and severe agricultural damage is likely or imminent (Ak et al. 2019; Damos et al. 2020). As this pest continues its outward expansion from the originally-established populations in Europe, knowledge of which populations are present and spreading may improve our understanding of the movement of *H. halys*, particularly in locations that experience high levels of economic damage in southern and eastern Europe and western Asia.

Mitochondrial DNA sequence data have frequently been used to trace the origin and spread of invasive insect species (Grapputo et al. 2005; Corin et al. 2007; Auger-Rozenberg et al. 2012; Chapman et al. 2015). The mitochondrial Cytochrome Oxidase I (COI) gene has shown utility in species identification and separation of genetic lineages (Bucklin et al. 2011; Stephens et al. 2011), in particular as it relates to reconstructing routes of invasion (Auger-Rozenberg et al. 2012; Chapman et al. 2015). Although the Cytochrome Oxidase II (COII) gene of *H. halys* has also been sequenced (Xu et al. 2014; Cesari et al. 2015, 2018; Yan et al. 2021), the COI gene has been used more extensively in the characterisation of the invasion history, diversity and identity of *H. halys* haplotypes in both native and invaded regions (Gariepy et al. 2014, 2015; Cesari et al. 2015, 2018; Zhu et al. 2016; Morrison et al. 2017; Valentin et al. 2017; Lee et al. 2018; Horwood et al. 2019; Schuler et al. 2020; Yan et al. 2021). Based on the COI haplotype analysis of *H. halys*, it has been suggested that multiple invasion events took place in the initial / early stages of invasion in Europe (2007–2012), with the population in Switzerland (primarily haplotype H3 and H8) resulting from the establishment of individuals that arrived directly from China; the population in Italy (primarily H1) resulting from the establishment of individuals from the invasive population in the USA; and the population in Greece (predominantly haplotype H33) resulting from a separate establishment of *H. halys* from China (Cesari et al. 2015; Gariepy et al. 2015; Valentin et al. 2017). The continued and consistent detection of one or more of these same haplotypes in Europe following the initial invasion (e.g. H1, H3, H8 and H33; Cesari et al. 2015, 2018; Gariepy et al. 2015; Morrison et al. 2017; Šapina and Jelaska 2018; Schuler et al. 2020; Yan et al. 2021), largely supports the occurrence of a bridgehead effect, wherein particularly successful invasive populations have given rise to secondary invasions in other locations (Lombaert et al. 2010; Lawson Handley et al. 2011). However, in Italy and Greece, subsequent introductions directly from Asia may have also taken place between 2013 and 2019, as a combined total of 20 additional haplotypes (including 14 previously undescribed haplotypes) have been reported in more recent studies in these two countries (Morrison et al. 2017; Cesari et al. 2018; Schuler et al. 2020). To date, none of these haplotypes is known from other invaded countries, indicating that spread through the bridgehead effect has not occurred. In addition, the majority of the new haplotypes have not yet been described from Asia and their origin remains unknown.

Halyomorpha halys COI haplotypes have been reported from Austria, Croatia, France, Georgia, Greece, Hungary, Italy, Romania, Serbia, Slovenia, Switzerland and

Turkey (Gariepy et al. 2014, 2015; Cesari et al. 2015, 2018; Morrison et al. 2017; Šapina and Jelaska 2018; Schuler et al. 2020; Yan et al. 2021). In some cases, the reports are based on a few specimens from a single location within a given country, based on their availability for study (e.g. Croatia, Serbia and Turkey). Although they may not provide a thorough account of the haplotype composition in a given country, these reports are valuable, as they provide information on the presence of a given haplotype and can continue to be built upon as additional samples become available to generate a more complete picture of the haplotype composition. *Halyomorpha halys* haplotype composition in more recently invaded areas of southern and eastern Europe, as well as western and central Asia, remain relatively undescribed. Identification of the haplotypes that are present in these areas may provide insight into the origin and spread of *H. halys* in these regions, particularly as it relates to secondary invasions from other countries in the invaded range. To address this, the DNA barcode region of the COI gene was analysed from samples collected from Georgia (including Abkhazia), Kazakhstan, Russia, Serbia and Ukraine and compared to the haplotype framework generated in previous studies (Gariepy et al. 2014, 2015; Cesari et al. 2015, 2018; Zhu et al. 2016; Morrison et al. 2017; Schuler et al. 2020; Yan et al. 2021). The present study builds upon a recent haplotype report from Georgia and Serbia (Yan et al. 2021), but with more extensive sampling and assessment of the haplotype composition in these two countries and characterises haplotype composition from Russia, Ukraine and Kazakhstan, which have not previously been assessed. Based on the characterisation of haplotypes from these areas and comparison with global records of *H. halys* haplotypes, we speculate on the pattern of diversity and spread and discuss the possible pathways of entry into these regions.

Methods

Acquisition of material

Halyomorpha halys adults were field-collected (by sweep net, hand-picking from vegetation or in pheromone traps) between 2016 and 2019 from locations with recently-established populations in Georgia (including Abkhazia; 2016–2018; n = 293), Kazakhstan (2017; n = 11), Russia (2016, 2018, 2019; n = 202), Serbia (2018; n = 129) and Ukraine (2017; n = 11) (Fig. 1). Note that Abkhazia is a disputed territory within the Caucasus; however, for the purpose of this study, we considered Abkhazia as located within Georgia, based solely on the geographic continuity of the agricultural landscape in this area. As such, throughout this manuscript, samples from Georgia and the disputed territory of Abkhazia will collectively be referred to as samples from Georgia. Complete specimen and collection data are publicly available at www.boldsystems.org (Project *Halyomorpha halys* in eastern Europe and Eurasia, EEUR) and are summarized in Suppl. material 1: Table S1. Individual insects were stored in 95% ethanol for subsequent molecular analysis.
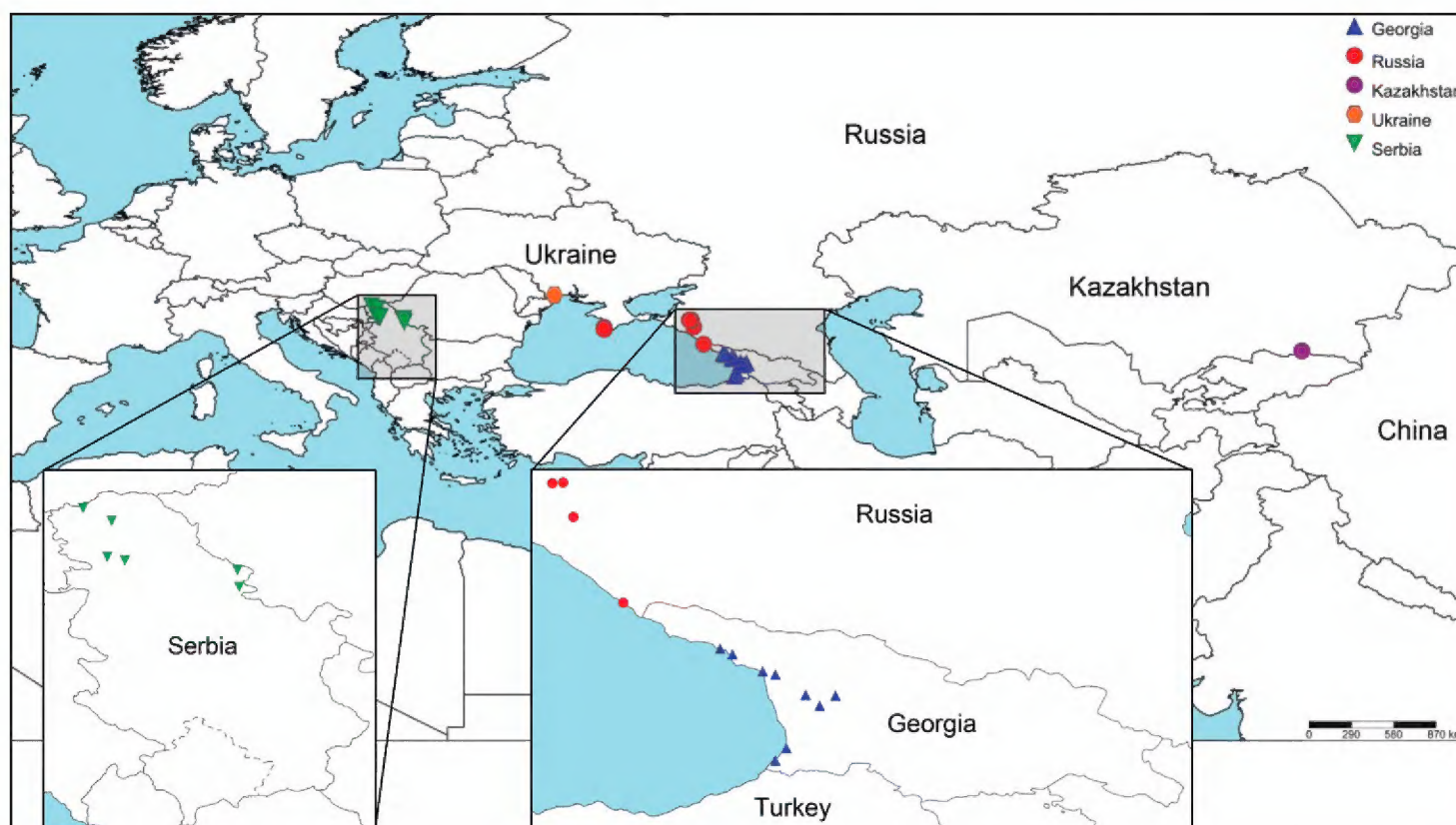
Figure 1. Map of *Halyomorpha halys* collection locations in Serbia, Ukraine, Russia, Georgia and Kazakhstan.

DNA extraction, amplification and sequencing

As described by Gariepy et al. (2014), a single leg was carefully removed from each insect using flame-sterilised forceps and placed in an individual 200 µl well of a 96-well microplate, along with 2 µl of proteinase K (20 mg/ml) and 100 µl of 5% Chelex 100 Molecular Grade Resin (Bio-Rad Laboratories, Hercules, CA, USA). A negative extraction control containing the Chelex and Proteinase K solutions, but no insect tissue, was included in each microplate. Sealed microplates were incubated overnight at 55 °C, followed by 10 min at 99 °C. Samples were centrifuged at 5800 g for 5 min to pellet the Chelex solution and 50 µl of supernatant (containing DNA) was transferred to wells in a new plate, taking care not to transfer the Chelex residue along with the sample. Microplates containing the extracted DNA were stored at – 20 °C until further analysis.

PCRs were performed in a 25 µl volume containing 0.125 µl of Taq Platinum, 2.5 µl of 10× PCR buffer, 1.25 µl of 50 mM $MgCl_2$, 0.125 µl of 10 µM dNTPs (Invitrogen, Carlsbad, CA, USA), 0.25 µl of 10 µM forward and reverse primer (respectively), 19.5 µl ddH_2O and 1 µl of template DNA. A 658-bp sequence of the mitochondrial gene Cytochrome C oxidase subunit I (COI) was amplified by PCR using primers LCO1490 and HCO2198 (Folmer et al. 1994). Thermocycling conditions included initial denaturation at 94 °C for 1 min, followed by five cycles of 94 °C for 30 s, annealing at 45 °C for 40 s, extension at 72 °C for 1 min, followed by another 35 cycles of 94 °C for 30 s, 51 °C for 40 s and 72 °C for 1 min and a final extension period of 5 min at 72 °C.

PCR products were visualised with a QIAxcel Advanced automated capillary electrophoresis system (Qiagen, Hilden, Germany) using the DNA screening cartridge

and method AL320. Results were scored with QIAXCEL SCREENGEL Software (version 1.2.0) and only those samples of the expected fragment size with a signal strength exceeding 0.1 relative fluorescent units were scored as positive.

Samples, scored as positive, were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA), following the manufacturer's instructions. Purified PCR products were bidirectionally sequenced on an ABI 3730 DNA Analyser at the Robarts Research Institute (London Regional Genomics Centre, ON, Canada). Forward and reverse sequences were assembled and edited using CODONCODE ALIGNER, version 9.0.1 (Codon-Code Corporation, Centreville, MA, USA). Sequence data and trace files were uploaded to the Barcode of Life Datasystems (BOLD; www.boldsystems.org) in the Project *Halyomorpha halys* in eastern Europe and Eurasia (EEUR).

Haplotype and nucleotide diversity measures

Samples were grouped, based on their country of collection (Georgia, Kazakhstan, Russia, Serbia and Ukraine) and standard measures of diversity were calculated for each group using DnaSP v.5.10.01 (Librado and Rozas 2009), including number of haplotypes, haplotype diversity (h, the probability that two randomly-selected haplotypes are different; Nei 1987) and nucleotide diversity (π, the average number of nucleotide differences per site between two randomly-selected DNA sequences; Nei and Li 1979).

Frequency and distribution of haplotypes of *Halyomorpha halys*

Samples were grouped, based on their country of collection (Serbia, Ukraine, Russia, Georgia and Kazakhstan) and the proportion of each haplotype within each group (i.e. country) was calculated in order to obtain a representation of the haplotype composition.

Additionally, based on current data and previous publications (e.g. Gariepy et al. 2014, 2015; Cesari et al. 2015, 2018; Šapina and Jelaska et al. 2018; Schuler et al. 2020; Yan et al. 2021), the number of COI haplotypes and the identity of dominant haplotypes from invaded European and central Asian countries were tallied and used to generate an overview of the trends and dominant haplotypes in these areas.

Results

Haplotype and nucleotide diversity measures

Genetic diversity measures for *H. halys* collected in Georgia, Kazakhstan, Serbia, Russia and Ukraine are shown in Table 2. For all samples combined, there were five haplotypes with a total of nine polymorphic sites. Overall haplotype and nucleotide diversity was 0.223 ± 0.021 (mean ± SD) and 0.00052 ± 0.00007 (mean ± SD), respectively. Haplotype and nucleotide diversity was zero in samples collected from Russia, Georgia

Table 2. Genetic diversity measures for *Halyomorpha halys*.

Country	Number of specimens	Number of Polymorphic sites	Number of haplotypes	Haplotype diversity (*h*) (mean ± SD)	Nucleotide diversity (*π*)(mean ± SD)
Kazakhstan	11	1	2	0.327 ± 0.153	0.0005 ± 0.00023
Georgia	293	0	1	0	0
Russia	202	0	1	0	0
Serbia	129	9	5	0.620 ± 0.022	0.00163 ± 0.00019
Ukraine	11	0	1	0	0
All	**646**	**9**	**5**	**0.223 ± 0.021**	**0.00052 ± 0.00007**
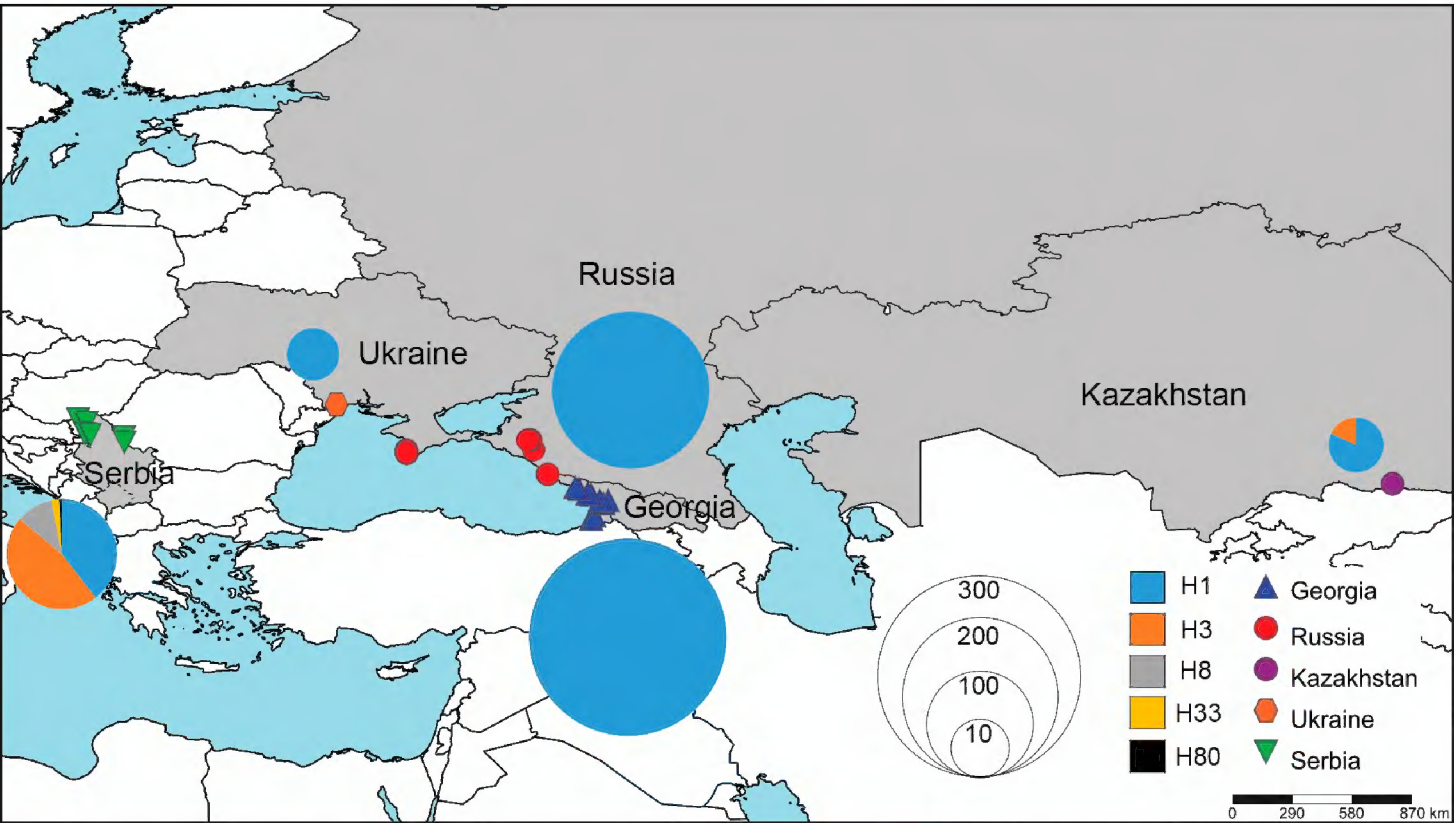
Figure 2. Map of *Halyomorpha halys* collection locations, with the COI haplotype frequency shown in pie charts sized proportionally to the sample size from each country.

and Ukraine, as only a single haplotype was observed. Serbia had the most diverse population observed in the present study, with five haplotypes recorded and showed the highest haplotype and nucleotide diversity (Table 2).

Frequency and distribution of haplotypes of *Halyomorpha halys*

A total of 646 samples were analysed and a 658-bp fragment of the DNA barcoding region of the COI gene was generated (Genbank Accession numbers MZ871818 - MZ872463). Collectively, five COI haplotypes (H1, H3, H8, H33 and H80) were identified. The majority of the samples were identified as haplotype H1 (87.6%), followed by H3 (9.6%), H8 (2.1%), H33 (0.5%) and H80 (0.2%).

The proportion of haplotypes from each country is shown Fig. 2 and Table 3. Haplotype H1 was recorded in all five countries and was either the dominant haplotype or the only haplotype in four of the five countries: Georgia (100%), Kazakhstan (82%), Russia (100%) and Ukraine (100%) (Table 3). In Serbia, H1 was the second

Table 3. Proportion (%) of each COI haplotype from *Halyomorpha halys* collected in Eastern Europe and Eurasia.

Haplotype	Georgia (n = 293)	Kazakhstan (n = 11)	Serbia (n = 129)	Russia (n = 202)	Ukraine (n = 11)
H1	100	82	39.5	100	100
H3	0	18	46.5	0	0
H8	0	0	10.9	0	0
H33	0	0	2.3	0	0
H80	0	0	0.8	0	0

Figure 3. Trends in the number of cytochrome oxidase I (COI) haplotypes in the invasive range of *Halyomorpha halys* in Eurasia.

most common haplotype, but nonetheless, represented 39.5% of the haplotype composition, making it a major contributor to the haplotype composition. H3 was the dominant haplotype from Serbia, representing 46.5% of the haplotype composition and was also recorded from Kazakhstan (18% of the haplotype composition). Three additional haplotypes were recorded from Serbia: H8 (10.9%), H33 (2.3%) and H80 (0.8%) (Fig. 2 and Table 3). The known global distribution of these haplotypes in their native and invasive ranges is presented in Table 4.

An overview of the number of haplotypes and the dominant haplotypes in non-native countries in Eurasia is presented in Table 5. The haplotype composition in the majority of countries in this invaded region consists of a single haplotype or relatively few haplotypes (≤ 4) (Fig. 3). Only four countries demonstrate a moderate (5–7 haplotypes: Switzerland and Serbia) to high (> 8 haplotypes: Italy and Greece) number of reported haplotypes (Fig. 3). All countries with *H. halys* COI haplotype data in Eurasia are dominated or co-dominated by H1, with the exception of Switzerland and France, which are dominated by H3 (Table 5, Fig. 4).

Discussion

Haplotype and nucleotide diversity

Invasive species typically have reduced genetic variation due to the occurrence of genetic bottlenecks upon colonisation of new locations (Fauvergue et al. 2012). Nonetheless,

Table 4. Overview of *Halyomorpha halys* haplotypes found in Eastern Europe and Central Asia, and their known global distribution in the native Asian range and in the invaded ranges. Countries in **bold** lettering indicate new records for a given haplotype.

Haplotype		Known distribution within native and invasive ranges	Original haplotype and/or location descriptions
H1	Native	China	Gariepy et al. 2014
	Invasive	USA, Canada	Gariepy et al. 2014
		Switzerland, France, Greece, Hungary	Gariepy et al. 2015
		Italy	Cesari et al. 2015
		Croatia	Šapina and Jelaska 2018
		Romania	Cesari et al. 2018
		Austria, Serbia, Slovenia, Georgia, Turkey, Chile	Yan et al. 2021
		Kazakhstan, **Russia**, **Ukraine**	Present study
H3	Native	China	Gariepy et al. 2014
	Invasive	Switzerland	Gariepy et al. 2014
		France, Hungary, Greece	Gariepy et al. 2015
		Italy	Cesari et al. 2015
		Austria, Serbia, Slovenia, Chile	Yan et al. 2021
		Kazakhstan	Present study
H8	Native	Unknown	-
	Invasive	Switzerland, France	Gariepy et al. 2014
		Italy	Cesari et al. 2015
		Austria	Yan et al. 2021
		Serbia	Present study
H33	Native	China	Valentin et al. 2017
	Invasive	Greece	Gariepy et al. 2015
		Italy	Cesari et al. 2018
		Serbia	Present study
H80	Native	China	Zhu et al. 2016
	Invasive	Italy	Cesari et al. 2018
		Serbia	Present study

some species have become very successful colonisers despite strong founder effects (Sax and Brown 2000), particularly when multiple introductions from different locations contribute to enhanced diversity (Miller et al. 2005; Dlugosch and Parker 2008; Lawson Handley et al. 2011). Previous studies in Europe have suggested establishment of *H. halys* from multiple sources, including the direct establishment of Asian populations, as well secondary invasions via previously-established populations through the bridgehead effect (Gariepy et al. 2015; Valentin et al. 2017; Schuler et al. 2020). A low genetic diversity in invasive *H. halys* has been observed in European countries where only a single haplotype is present (e.g. Romania; Cesari et al. 2018; Yan et al. 2021) or where one haplotype is dominant amongst a small number of haplotypes (e.g. Switzerland, Hungary and France; Gariepy et al. 2015). In these countries, *H. halys* populations have established either directly from Asia (e.g. from China to Switzerland) or by secondary invasion from neighbouring European countries (e.g. from Switzerland to France), with haplotype diversity (*h*) ranging from 0 to 0.27 and nucleotide diversity (π) ranging from 0 to 0.0008 (Gariepy et al. 2014, 2015; Cesari et al. 2018; Yan et al. 2021).
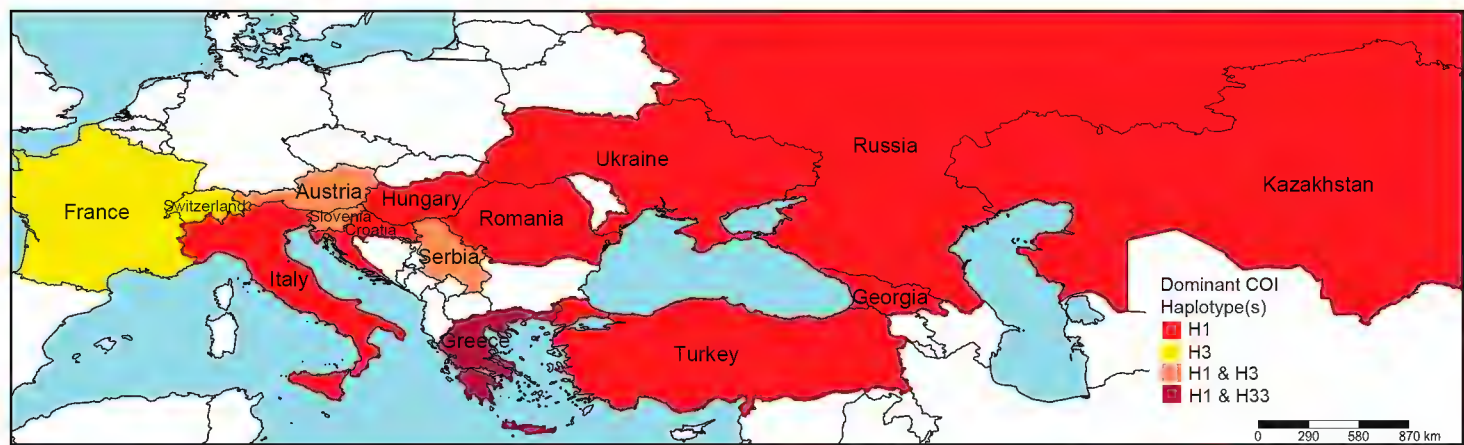
Figure 4. Trends in the distribution of dominant cytochrome oxidase I (COI) haplotypes in the invasive range of *Halyomorpha halys* in Eurasia.

In contrast to other invaded countries in Europe, *H. halys* populations in Italy and Greece are substantially more diverse (h = 0.702–0.724; π = 0.0036–0.0054; Gariepy et al. 2015; Cesari et al. 2018), with establishments that have originated from multiple source locations, including directly from China, Japan and Korea and/or via the bridgehead effect from established populations in the USA (Cesari et al. 2015, 2018; Gariepy et al. 2015; Morrison et al. 2017; Valentin et al. 2017; Schuler et al. 2020).

In the present study, the overall haplotype (h = 0.223) and nucleotide diversity (π = 0.00052) of the COI barcode region was relatively low and is consistent with the values mentioned above from previous studies in most European countries (excluding Italy and Greece). However, diversity spanned a broad range, with no diversity in samples from Russia, Ukraine and Georgia (where a single haplotype was found) to haplotype and nucleotide diversity values of 0.62 and 0.00163 (respectively) from samples collected in Serbia (where a total of five haplotypes were found). Yan et al. (2021) found similar results in terms of a lack of haplotype diversity in their samples collected in Georgia, where a single COI haplotype was found. Our larger number of samples from several additional areas in Georgia provides a more thorough assessment of the populations in this region and confirms the observations by Yan et al. (2021) in that a single haplotype is (currently) present in this country. The population from Kazakhstan yielded diversity values similar to those reported in European countries with < 5 haplotypes (Fig. 3); however, it is based on a small sample size (n = 11) collected from a single site and, therefore, may not accurately represent the diversity in that country. Goodall-Copestake et al. (2012) recommend sample sizes ≥ 25 for accurate comparisons of population-level COI diversity; nonetheless, our limited samples from Kazakhstan provide a baseline dataset from a previously-unrepresented country that can be built upon with further sampling. This may also be the case with our samples from Ukraine, as the sample size was small and originated from a single region (Odessa). However, given the fact that current and previous studies in neighbouring countries (Romania, Hungary and Russia) have also shown little to no haplotype diversity, it is not surprising that similar results were found in Ukraine in the present study, despite the small sample size. Nonetheless, more thorough sampling in additional,

Table 5. Summary of the number of samples analyzed, the number of haplotypes detected, and the proportion of the dominant haplotypes (H1, H3, H33) in the overall haplotype composition in invaded countries in Eurasia.

Country	Reference	Number of samples	Number of haplotypes	Proportion (%)			
				H1	H3	H33	Other
Austria	Yan et al. 2021	16	4	25	50	0	12.5
Croatia	Šapina and Jelaska 2018	2	1	100	0	0	0
France	Gariepy et al. 2015	139	3	0.6	98	0	1.4
Georgia	Present study	293	1	100	0	0	0
Greece	Gariepy et al. 2015	57	7	32	7	40	≤14
	Morrison et al. 2017	195	11	32	4.1	46.2	0.5–8.2
	Cesari et al. 2018	10	3	20	0	60	20
Hungary	Gariepy et al. 2015	84	2	99	1	0	0
	Morrison et al. 2017	194	2	99.5	0	0	0.5
	Yan et al. 2021	92	3	93	5	0	2
Kazakhstan	Present study	11	2	82	18	0	0
Italy	Cesari et al. 2015	42	3	76	21	0	3
	Morrison et al. 2017*	187	11	60	22.5	0	0.5–3
	Cesari et al. 2018	212	13	50	12	0	0.5–15
	Schuler et al. 2020	162	15	53	15	1	0.5–10
Romania	Cesari et al. 2018	8	1	100	0	0	0
	Yan et al. 2021	23	1	100	0	0	0
Russia	Present study	202	1	100	0	0	0
Serbia	Present study	129	5	39.5	46.5	2.3	0.8–11
Slovenia	Yan et al. 2021	16	3	69	25	0	6
Switzerland	Gariepy et al. 2015	225	4	0.9	85	0	0.4–14
	Morrison et al. 2017	110	4	1.8	89.5	0	2.6–6
Turkey	Yan et al. 2021	11	1	100	0	0	0
Ukraine	Present study	11	1	100	0	0	0

*Original data were presented separately from three regions within Italy (Emilia-Romagna, Veneto, and Piedmont); the values reported here were estimated by combining the values for each region. See Morrison et al. (2017) for detailed breakdown of haplotype frequencies in different regions of Italy.

geographically-diverse locations would help to confirm this observation and/or record changes in diversity over time as the invasion progresses.

The most diverse population in the present study was recorded from Serbia. Although Yan et al. (2021) found lower values for haplotype and nucleotide diversity from their Serbian samples, their values were based on nine specimens collected from a single site, which likely underestimated the actual diversity in Serbia (as per Goodall-Copestake et al. 2012). This is supported by the fact that Yan et al. (2021) recorded only two COI haplotypes, whereas the present study recorded five haplotypes and consisted of a much larger sample size from several locations (Fig. 1; Table 5). The diversity measures that we recorded from Serbia (h = 0.62; π = 0.00163) are just slightly lower than those reported in Italy and Greece (h = 0.702–0.724; π = 0.0036–0.0054; Gariepy et al. 2015; Cesari et al. 2018), where several haplotypes from multiple source populations (including China, Japan, Korea and USA) are known to occur (Gariepy et al. 2015; Valentin et al. 2017; Cesari et al. 2018). As will be discussed more thoroughly below, we suspect that *H. halys* populations in Serbia are derived from multiple source populations from more

than one of the surrounding European countries, resulting in diversity levels more similar to those found in countries with multiple sources of invasion (e.g. Italy and Greece).

Frequency and distribution of COI haplotypes and potential sources of *Halyomorpha halys*

Based on the present study and the collective dataset available in literature for *H. halys* (see Table 5), the occurrence of COI haplotypes is fairly uniform across most of eastern Europe and central Asia (Figs 3 and 4). In most countries, only a single haplotype (H1) is present (e.g. Croatia, Romania, Ukraine, Turkey, Russia and Georgia) or is dominant alongside a minor contributing haplotype (typically H3; Kazakhstan and Hungary) (Figs 3 and 4). The exception in the present study is Serbia, where a total of five haplotypes were detected, including two dominant haplotypes (H1 and H3) and three additional haplotypes (H8, H33 and H80), two of which were minor contributors (< 5%; H33 and H80). A similar exception in previous studies was observed in Greece, where two dominant haplotypes are also known (H1 and H33; Fig. 4 and Table 5), along with several minor contributing haplotypes (H3, H13, H22, H30, H31, H32, H158, H159 and H160; Gariepy et al. 2015; Morrison et al. 2017).

Greece and Italy are known hotspots of invasive *H. halys* haplotypes, with 11 (Gariepy et al. 2015; Morrison et al. 2017) and 20 COI haplotypes (Morrison et al. 2017; Cesari et al. 2018; Schuler et al. 2020), respectively. Further, within the invaded range, many of these haplotypes are unique to these two countries (i.e. are not found elsewhere in Europe). For example, in the native and invasive ranges of *H. halys*, H80 was previously only known from Shandong Province in China (Zhu et al. 2016) and from northern Italy (Cesari et al. 2018; Schuler et al. 2020). However, in the present study we found this haplotype at very low levels in Serbia. Given the relatively low occurrence of this haplotype in Italy (Cesari et al. 2018), it is difficult to speculate whether the source of H80 is the result of movement and spread of H80 from Italy or whether it is a separate establishment originating from China. Similarly, H33 was previously only known from Greece (Gariepy et al. 2015; Morrison et al. 2017), but was recently detected for the first time in Italy by Schuler et al. (2020). Although H33 is known from China (Shanxi, Shaanxi and Anhui Provinces; Zhu et al. 2016; Valentin et al. 2017; Cesari et al. 2018), Schuler et al. (2020) suggest that the movement and spread of this haplotype from the already-established population in Greece is more likely the source of H33 in Italy, especially given the prevalence and persistence of this haplotype in Greece (Gariepy et al. 2015; Morrison et al. 2017). The detection of H33 in Serbia in the present study also suggests movement and spread of H33 through secondary invasion from Greece; however, it is unclear whether it is due to passive dispersal or associated with commercial trade and travel (Konjević 2020). As the occurrence of *H. halys* haplotypes in most of the countries that share a border with Serbia and Greece has not yet been investigated, it would be important to determine the occurrence of this (and other) haplotype(s) in such locations where *H. halys* is also known

to occur (Table 1), in particular Bulgaria, North Macedonia, Albania, Montenegro and Bosnia and Herzegovina. This may provide insight as to whether the distribution of H33 is widespread or continuous across the Balkan countries or whether it is primarily in urban centres associated with commercial trade and travel. All of our samples were collected in north and north-eastern Serbia (Fig. 1), which is bordered by countries where a single haplotype (H1) is known or dominant (e.g. Croatia, Hungary and Romania; Fig. 4) and where H33 and H80 have not been reported. The movement of H33 and H80 into Serbia is, therefore, unlikely from this direction – neither through natural dispersal nor from commercial trade or travel. As such, sampling in the southern portion of Serbia would provide a more thorough account of the haplotype distribution across the entire country and provide insight on the movement and spread of H33 and H80, particularly from countries along the southern border of Serbia. In contrast, the movement of haplotype H1 likely occurred from the spread of this haplotype from neighbouring countries to the west (Italy), north (Hungary) and/or northeast (Romania), where H1 is dominant. *Halyomorpha halys* populations in Serbia were first observed in areas near or along the Serbian-Romanian border (Šeat 2015) and in close proximity to the railway line that connects Bucharest, Romania with Belgrade, Serbia (Musolin et al. 2018). Thus, the trapping and interception records support the secondary invasion of H1 from neighbouring eastern European countries (as opposed to separate establishment events from China), possibly associated with railway travel or movement of commodities on railway cars, as suggested by Musolin et al. (2018). In terms of H3, which was dominant in Serbia, the prevalence of this haplotype in western Europe (in particular Switzerland, France and Austria), as well as the known presence (albeit at low levels) in Hungary, could indicate movement and spread from this direction. Similarly, H8 is the second most common haplotype in Switzerland and is also present (at lower levels) in France and Italy (Gariepy et al. 2014, 2015; Cesari et al. 2018; Schuler et al. 2020), suggesting the direction of movement of H8 is likely from western Europe to Serbia. In neighbouring Croatia, only H1 has been reported by Šapina and Jelaska (2018), but this is only based on two specimens; further analysis of samples in Croatia would help clarify the occurrence and diversity of additional haplotypes, in particular H3 and H8, which would be interesting in terms of evaluating the spread of haplotypes from this direction. Serbia is located directly at the centre of the invasive range of *H. halys*, surrounded by countries with different haplotype compositions. Although we cannot exclude the possibility of separate invasion(s) from China, the likely scenario (based on location, haplotype and trapping data) is that *H. halys* is entering Serbia from more than one direction simultaneously (through natural dispersal, via commercial/horticultural trade and/or travel). Although largely speculative, H1 may have initially arrived in Serbia from neighbouring countries to the east (possibly with additional invasions from other directions, given that surrounding countries all have a high proportion of H1), with H3 and H8 arriving from the western European countries and H33 and H80 from Greece and Italy.

The first established population of *H. halys* in the area of eastern Europe and central Asia occurred in Sochi City, Russia in 2013–2014 and at the time the pest was

not present in neighbouring countries (see Table 1). The fact that this establishment was geographically disconnected from the rest of the invaded range in Europe suggests that secondary invasion from natural dispersal of the pest is unlikely (Musolin et al. 2018). Although the source of *H. halys* could be the result of a separate introduction from Asia, a secondary invasion via the bridgehead effect is more likely, based on the timing and location of the arrival of *H. halys* and the events surrounding its establishment. Musolin et al. (2018) suggest that the pest was accidentally introduced from Italy or Greece with infested plant material that was used in massive landscaping efforts associated with the 2014 Winter Olympic Games hosted in Sochi; plants from northern Italy were regularly imported in 2012–2013 due to a similarity between the climates of the two regions and low availability of local stock to meet the landscaping demands leading up to the Olympic Games. This coincides with the occurrence of high population levels of *H. halys* in northern Italy and reports of economic damage to crops (Pansa et al. 2013; Maistrello et al. 2014). The *H. halys* population in Italy is predominantly H1 in most regions, in particular Emilia Romagna and Lombardy (Cesari et al. 2015; Morrison et al. 2017; Cesari et al. 2018; Schuler et al. 2020). This information, combined with the fact that the present study demonstrated that H1 is the only haplotype in Russia (based on > 200 specimens), lends support to the theory proposed by Musolin et al. (2018) that *H. halys* in Sochi may have originated from locations in Italy where H1 is dominant. From the focal point of Sochi City in Russia, *H. halys* may have dispersed to other regions in the Caucasus. However, populations of H1 in some of these locations (e.g. Sevastopol), as well as neighbouring locations in Ukraine (Odessa) and Georgia (Abkhazia, Adjara and Samegrelo), could be due to the spread of *H. halys* from other locations associated with the movement of commercial goods, as all of these cities are important seaports within the region. A similar concern in Australia was also flagged as a threat, when a significant number of live *H. halys* were intercepted in shipments arriving from Italy; haplotype H1 and H23 were both identified from shipments originating from Italy (Horwood et al. 2019), demonstrating how easily secondary invasion could occur, even over long distances.

In Kazakhstan, *H. halys* was first reported in 2016 in Almaty and establishment was confirmed when populations continued to expand in the area in 2017 and 2018 (Esenbekova 2017; Temreshev et al. 2018). Although the present study consisted of a limited number of samples from Almaty, Kazakhstan (n = 11), two haplotypes (H1 and H3) were detected, with H1 being dominant. Almaty shares a border with Xinjiang Province in China; however, *H. halys* is not known to occur in Xinjiang or Qinghai (Yu and Zhang 2007), indicating that the source of this pest in Kazakhstan is not from natural dispersal near or along the Kazakhstan-China border. Similarly, Almaty is far removed from the distribution of *H. halys* in the Caucasus region, suggesting that natural dispersal from this region is not responsible for the occurrence of the pest in Kazakhstan. However, Almaty is the major commercial centre of Kazakhstan and, as such, we speculate that commercial trade (with China, Russia and/or other European countries) would be the source of H1 and H3 in this country. Kazakhstan has a relatively low number of invasive alien species currently recorded; however, the recent rise in international trade and oil, gas and mining

development in Kazakhstan, Turkmenistan, and Uzbekistan will also likely result in an increase of invasive species in these countries (Turbelin et al. 2017). Given Kazakhstan's geographic location (i.e. directly between the invasive range of *H. halys* in Europe / Russia and the native range in China), its importance as the hub of international trade in central Asia and its position as a major transportation hub linking China to Russia and western Europe by air, rail, road and sea (Selmier 2020), we speculate that *H. halys* invasion from Europe, Russia and China are all very likely. Another invasive hemipteran, *Leptoglossus occidentalis* Heidemann (Hemiptera: Coreidae), was recently discovered in Kazakhstan (Barclay and Nikolaeva 2018) and is similarly far-removed from other known established populations. Its arrival is likely due to passive transportation of adults as stowaways in cargo or through nursery trade (Barclay and Nikolaeva 2018), which is likely the same pathway of entry for *H. halys* in this region. The collection and COI haplotype analysis of additional *H. halys* samples from this area would provide a more thorough documentation of the haplotype diversity in Kazakhstan. However, given its separation from the centre of other *H. halys* invasions in Eurasia and without interception records to corroborate potential pathways of entry, additional COI haplotype analysis will only tell us which countries have a similar haplotype composition and is unlikely to clarify whether the pathway of entry is directly from the native range in Asia or whether it is the result of a secondary invasion via Europe, Russia or some combination thereof (but see future directions below).

Future directions

The present study focused solely on the COI gene, as this gene has shown reliability in terms of revealing geographic patterning (O'Loughlin et al. 2008; Valade et al. 2009) and has been widely utilised in haplotype studies on *H. halys* and has the most comprehensive, publicly-available global haplotype network available for this species. However, more in-depth multilocus analysis, based on microsatellite DNA or high-resolution genomic data (e.g. single nucleotide polymorphisms, SNPs; restriction site associated DNA sequencing, RADseq), may reveal additional patterns regarding invasion pathways (Garnas et al. 2016; Sunde et al. 2020). As techniques for generating high-resolution genomic data become more mainstream in ecological studies (see Andrews et al. 2016), future research that makes use of these newer techniques to investigate the global diversity of *H. halys* may provide a more fine-tuned interpretation of patterns of dispersal, in particular in terms of dissecting pathways of entry from the area of pest origin versus movement and spread of already-established populations to new locations.

Conclusions

The results, presented here, provide haplotype coverage of previously uninvestigated or under-investigated regions along the easternmost front of the invasion of *H. halys* in Eurasia. The evaluation of *H. halys* haplotypes along the eastern front of the invasion in Eurasia demonstrates the continued spread and successful establishment of haplo-

type H1 in newly-invaded areas, where it is often the only haplotype. Secondary invasions within Europe are likely responsible for the movement and spread of additional haplotypes (e.g. H3, H33 and H80) that are moving beyond their first detection and establishment points. In addition, countries at the centre of the invaded range, such as Serbia, are proving to be more diverse due to multiple invasion events from neighbouring countries with differing haplotype compositions.

Several gaps remain to be filled to generate a more complete picture of the haplotype composition across this region. For example, additional collection and haplotype analysis of *H. halys* is necessary in countries where the pest is known to occur, but where large-scale haplotype analysis has not yet been done. Further, in some locations, more thorough collections are warranted in order to more accurately estimate the haplotype composition and diversity across the entire range of the pest (e.g. Kazakhstan, Ukraine and Croatia). Despite the presence of multiple haplotypes in the invasive Eurasian range, H1 is clearly dominant (Fig. 4) and associated with the majority of recent invasions along the eastern front of the spread of *H. halys* in these areas. A number of examples exist in literature where the range expansion of an invasive insect species is primarily associated with one mitochondrial haplotype (e.g. Grapputo et al. 2005; Dittrich-Schröder et al. 2018; Brookes et al. 2020; Machado et al. 2020), with some populations contributing disproportionally to global spread (Garnas et al. 2016). The same pattern is observed in the spread of *H. halys*; haplotype H1 is now known from a total of 18 countries outside of its native range (Table 4) and is the dominant haplotype in the majority of these countries (Table 5). Interestingly, reports of serious agricultural damage are known primarily from those countries where H1 is prevalent (e.g. Georgia, Greece, Italy, Russia and Turkey; Maistrello et al. 2017; Bosco et al. 2018; Musolin et al. 2018; Ak et al. 2019; Damos et al. 2020). This prevalence of H1 across Eurasia could be linked to high levels of traffic and commercial trade between these countries (i.e. global connectivity of non-contiguous areas; Garnas et al. 2016) facilitating the spread of an already-established, dominant haplotype through the bridgehead effect (Lombaert et al. 2010). In addition, climatic factors in some regions may permit more rapid population growth (and increased agricultural impacts) due to multiple generations of any given successfully-established haplotype (Musolin et al. 2019; Stoeckli et al. 2020). However, variation in performance traits in invasive insect species can be an important predictor of their success in establishment and spread across a broad geographic range (Thompson et al. 2021). Future research investigating the different *H. halys* haplotypes would be of interest to determine whether there is any validity to the observation that H1 appears to be a more successful invader and to determine whether this success is linked to intraspecific variation in biological traits, such as phenology, thermal performance, flight capacity, overwintering survival or fecundity.

Acknowledgements

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Supplementary material 1

Table S1. Collection information and GPS coordinates

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Data type: excel table

Explanation note: Details on the collection locations.

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Link: https://doi.org/10.3897/neobiota.68.68915.suppl1